REMARKS

Review and reconsideration on the merits are requested.

With respect to the rejection of claims 6 and 7 under 35 U.S.C. § 112, second paragraph, "type" is deleted from claim 6 and thus claim 7 (claim 7 was amended February 4, 2003, to delete "type"). Withdrawal of the rejection is requested.

Applicant submits and attaches hereto References 1 to 3:

Reference 1: Y. Saga, T. Watanabe, K. Koyama, and T. Miyasaka, *Chem. Lett.*, 1998, 961-962.

Reference 2: Y. Saga, T. Watanabe, K. Koyama, and T. Miyasaka, *J. Phys. Chem.*, B 1999, 103, 234-238.

Reference 3: K. Koyama, T. Miyasaka, R. Needleman, and J. Lanyi, *Chem. Lett.*, 1999, 769-770.

Prior art: U.S. Patent 5,107,104 Miyasaka (Miyasaka); U.S. Patent 6,300,559 Ohmori (Ohmori); U.S. Patent 6,300,612 Yu (Yu), U.S. Patent 4,985,618 Inada (Inada).

The art rejections: claims 1-4 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Miyasaka in view of Ohmori.

Claims 5-7 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Miyasaka in view of Ohmori, further in view of Yu.

Claims 8, 10-12, 16 and 17 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Miyasaka in view of Ohmori and further in view of Inada.

Thus, all claims were rejected.

The Examiner's reading of the prior art and application of the prior art is set forth in the Action, and will not be repeated here except as necessary to an understanding of Applicant's traversal which is now presented.

With respect to the Amendment to claims 1, claims 1/2 are combined. Further, Applicant includes into claim 1 "said semiconductor sensitized by a dye primarily adsorbing a light to cause the generation of electrons and positive holes in said dye, and thereafter said semiconductor receiving and conveying said electrons or said holes," in order to explain "a semiconductor sensitized by a dye".

The limit "said semiconductor sensitized by a dye primarily adsorbing the light to generate electrons and positive holes in said dye and thereafter said semiconductor receiving and conveying said electrons or said holes," finds support at page 11, lines 24-29 of the specification.

The limit "wherein said ion-conductive electrolyte layer is free of redox species" finds support at page 3, lines 8-10 of the specification.

Applicants first address the rejection of claims 1-4 under 35 U.S.C. § 103(a) based on Miyasaka in view of Ohmori. This rejection is respectfully traversed.

Since the requisite features of the present invention are recited in claim 1, Applicant discusses only the patentability of claim 1 (Amended) in presenting the following traversal.

With respect to the rejection of claims 1-4, at page 3 of the Action, top, the Examiner states as follows:

> Miyasaka discloses a differential response light-receiving device having a
> semiconductor 2 comprising a semiconductor sensitized by a dye 3, an ion-

conductive electrode 6, and a counter electrode 5 (fig. 1; col. 4, lines 5-15). The device makes a time-differential response to light to output a current (fig. 4).

Applicant must respectfully disagree with the Examiner's characterization of Miyasaka for the reasons now set forth.

Claim 1 (Currently amended) of the present application calls for:

1.   (Currently amended) A differential response light-receiving device comprising: a semiconductor electrode comprising an electrically conductive layer and a photosensitive layer containing a semiconductor sensitized by a dye; an ion-conductive electrolyte layer; and a counter electrode, said differential response light-receiving device making time-differential response to quantity of light to output a photoelectric current, said semiconductor sensitized by a dye primarily adsorbing a light to cause the generation of electrons and positive holes in said dye, and thereafter said semiconductor receiving and conveying said electrons or said holes, wherein said ion-conductive electrolyte layer is free of redox species.

If the Examiner will refer to the present specification at page 11, it can be seen that in the semiconductor sensitized by a dye of the present invention, the dye absorbs light in the visible region, the near infrared region, etc., to result in the generation of electrons and positive holes in the dye, the semiconductor then receiving the electrons or the positive holes thus generated. See the specification at page 11, lines 26-29 and page 18, lines 2-4.

In contrast to the present invention, Miyasaka does not teach or suggest a photosensitive layer 20 containing semiconductor fine particles 21 sensitized by dyes 22 and an electrolyte material 23 penetrated into voids among the semiconductor fine particles, and ion-conductive electrolyte layer 30 and a counter electrically conductive layer 40 laminated in this order

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(underscore added); see the present specification at page 8, line 17-22 and Fig. 1. Rather,

Miyasaka merely discloses an electrically conductive electrode substrate 2 including SnO_2, ITO,

etc. (a film used herein) <u>coated with</u> an oriented film of photosensitive chromoprotein 3, a

biosubstance such as bacteriorodopsin, a counter electrode 5, and electrolyte 6 and a spacer 4 for

retaining electrolyte 6 (underscore added); see the present specification at col. 4, lines 5-11 and

lines 40-42 and at col. 5, lines 50-59 and Figs. 1 and 2.

In this regard, it should be appreciated that in a certain protein membrane, e.g., a film of

bacteriorodopsin, the sole protein in the purple membrane of *Halobaacterium salinarium*, as the

result of light-absorption by turning on and off of a light, the photoinduced trans-cis

isomerization of all-trans retinal (Vitamin A aldehyde), a chromophore bonded to Lys-216 via a

protonated Schiff base, contained in bacteriorodopsin causes proton transport from one side of

the membrane to the other side thereof (called a proton pump) (see col. 1, lines 17-28 and

References 1-2), particularly, Reference 2, page 234, left column, lines 2-27.

Thus, as opposed to the present invention, in the photoelectric transducer of Miyasaka,

the light-driven protein pump as such causes a pH change on the electrically conductive substrate

2 (a semiconductor electrode such as SnO_2 or ITO), resulting in a change of surface potential,

thereby generating a transient photocurrent on the semiconductor electrode 2 (see col. 6, lines

14-27 and References 1-3, particularly Fig. 8 of Reference 2).

In distinction, in accordance with the present invention, as discussed in the specification

at pages 7-8, light injected to a photosensitive layer excites a dye contained therein, and electrons

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are injected from the excited dye to a semiconductor through an external circuit, resulting in charge separation in the dye to generate a dye hole, thereby generating a transient current (see page 7, line 24 to page 8 of the specification).

Further, in accordance with the present invention, the term "a semiconductor sensitized by a dye" means a semiconductor having the same photosensitivity as that of a dye caused by injecting electrons from the excited dye to the semiconductor, which is quite different from the Miyasaka semiconductor sensing a pH change based on the photoisomerization of the chromophore contained in bacteriorodopsin..

Accordingly, one of ordinary skill in the art referring to Miyasaka, which teaches a photoelectric transducer comprising an oriented film of photosensitive chromoprotein 3, such as bacteriorodopsin containing all-trans retinal as a chromophore, where photoinduced trans-cis isomerization causes a pH change on the electrically conductive electrode substrate 2, resulting in a change of surface potential, thereby generating a transient photocurrent, would see that Miyasaka is silent regarding a semiconductor sensitized by a dye, and thus would not be motivated to reach the invention recited in claim 1 (amended), even considering Miyasaka in view of Ohmori.

In this regard, Applicants note the argument made at page 8 of the Amendment of February 4, 2003, at page 8, at lines 3-15, was meant to emphasize the difference in critical properties between the semiconductor sensitized by a dye of the present invention and the semiconductor sensitized by a chromophore (all-trans Vitamin A aldehyde) having a structure completely different from those of phthalocyanines in photoelectric current.

Applicant appreciates that the rejection of claims 1-4 is not based only on Miyasaka,

rather, is further in view of Ohmori, and now address Ohmori.

At page 3 of the Action, beginning at the paragraph bridging pages 3/4 of the Action, the

Examiner states with respect to Ohmori as follows:

> "Ohmori discloses a dye-sensitized stationary response light-sensitized
> device having a semiconductor electrode comprised of a transparent electrode 2
> and a photosensitive layer comprising a semiconductor 3 sensitized by a dye 4, an
> electrolyte layer 5 containing a redox species, and a counter electrode 6 (fig. 1;
> col. 1, lines 26-37; col. 4, lines 17-24)."

Applicants must, however, respectfully disagree with the Examiner's above

characterization of Ohmori for the following reasons.

Ohmori, in fact, discloses a dye-sensitized photoelectric conversion element having a

laminate structure comprising a transparent electrode 2, thin film 3 formed of semiconductor

(TiO_2) particles, a dye 4 adhering to a surface of the semiconductor film 3, an electrolyte layer 5,

and a counter electrode 6, which are disposed in this order on the other side of film 1 (see

Ohmori at col. 1, lines 26-31 in view of claim 1 and Fig. 1). This type of dye-sensitized

photoelectric conversion device is a dye-sensitized solar cell called a "Graetzel cell" and is

discussed at page 7, lines 4-18 of the specification, referring to, for instance, U.S. Patent

4,927,721.

Thus, in Ohmori an electrolyte layer 5 capable of forming redox species such as I^-/I^{3-} for

transporting electrical charge functions to continuously transport electrons from the counter

electrode 6 to dye holes, whereby the dye-sensitized photoelectric conversion device outputs a

stationary photoelectric current to act as conventional solar cell (see col. 4, lines 17-23 of

Ohmori and page 7, lines 12-15 of the present specification).

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Thus, although the gross function of a dye-sensitized photoelectric conversion element of Ohmori is the same as that of the present invention in the sense of the initial step to form dye holes in a dye by injecting electrons from the photoexcited dye to a semiconductor, the steps (mechanism) thereafter are completely different from those of the present invention, as explained above.

In contrast to Ohmori, one major distinguishing feature of the present invention over Ohmori lies in fact that the ion-conductive electrolyte layer is free of redox species, whereby the ion-conductive electrolyte layer does not have a function of supplying electrons to dye holes and/or a function of receiving electrons from a counter electrode (see page 7, lines 21-24 of the specification). Thus, the differential response light-receiving device of the present invention was obtained based on the finding that when light is injected to a dye-sensitized semiconductor free of redox species capable of transporting electrical charge, there is seen a transient current response necessary to form a certain amount of dye holes corresponding to the quantity of the light injected, different from the conventional dye-sensitized photoelectric cell of Ohmori (see page 8, lines 5-11 of the specification).

Accordingly, Applicant respectfully submits that one of ordinary skill in the art referring to Ohmori, which teaches the use of a separate electrode 2 and a thin semiconductor film 3, but is silent regarding an electrolyte having no redox species, would not be motivated to reach the present invention as recited in claim 1 (Amended), and clearly even if Miyasaka is combined with Ohmori, there is no motivation to reach the present invention as claimed.

With respect to dependent claims 3/1 and 4/3, Applicant submits that the patentability of such claims is clear from the above traversal regarding claim 1 (Amended), and requests withdrawal of the rejection.

With respect to the rejection of claims 5-7 under 35 U.S.C. § 103(a) based on Miyasaka in view of Ohmori and further in view of Yu, as discussed above, neither Miyasaka nor Ohmori disclose or suggest in combination a device having the features recited in claim 1 (Amended) of the present application. Thus, even if further combined with Yu, the subject matter of dependent claims 5/1, 6/5 and 7/6 is not suggested.

Withdrawal is requested.

Turning to the rejection of claims 8, 10-12, 16 and 17 under 35 U.S.C. § 103(a) based on Miyasaka in view of Ohmori and further in view of Inada, for the same basic reasons as advanced with respect to the earlier rejection further in view of Yu, Applicant submits that dependent claims 8/1, 10/8, 11/12, 12/11, 16/1 and 17/18 are allowable.

Withdrawal is requested.

Turning finally to the rejection of claims 9 and 13-15 under 35 U.S.C. § 103(a) based on Miyasaka in view of Ohmori and in view of Inada and further in view of Yu, for the reasons earlier advanced regarding the failure of disclosure in Miyasaka and Ohmori in view of Inada, Applicant submits that dependent claims 9/8, 13/10, 14/13 and 15/14 are allowable, and requests withdrawal of the rejection.
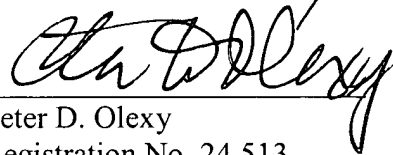
AMENDMENT UNDER 37 C.F.R. § 1.116
U.S. Appln. No. 09/963,419

Please consider this paper the same as a response to the rejection (final) of March 13,

2003.

The USPTO is directed and authorized to charge all required fees, except for the Issue

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Respectfully submitted,

Peter D. Olexy
Registration No. 24,513

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

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pH-Dependent Photocurrent Response from Bacteriorhodopsin at Electrode-Electrolyte Interfaces

Yoshitaka Saga, Tadashi Watanabe,* Koichi Koyama,[†] and Tsutomu Miyasaka[†]

Institute of Industrial Science, University of Tokyo, Roppongi, Minato-ku, Tokyo 106-8558

[†]*Ashigara Research Laboratories, Fuji Photo Film Co., Ltd., Minamiashigara 250-0193*

The transient photocurrent response of bacteriorhodopsin (bR) at electrode-electrolyte interfaces showed a drastic pH dependence, reversing at a low pH. The ratio of the photocurrent by turning the light off to that at the onset of illumination, I_{on}/I_{on}, decreased systematically from 0.92 to 0 by raising pH. These results can neatly be correlated with the proton release/uptake sequence in the bR photocycle.

Recently, the photoelectric response of bacteriorhodopsin (bR), the light-driven proton pump of *Halobacterium salinarium*,[1,2] has attracted much attention in the electrochemical field.[3-6] Irradiation to bR on an electrode generates a transient photocurrent by turning on and off of light, but the origin of this unique photoresponse has not been thoroughly unraveled. Following a preliminary work[7] suggesting that the photoresponse originates in the proton release/uptake by bR molecules, we have systematically examined here the effect of electrolyte pH on the photocurrent pattern, and established a clear correlation between the photoresponse and the bR photocycle.

Photocurrent measurements were done in a manner similar to that described elsewhere.[7] A suspension of purple membranes was deposited on an SnO_2 plate (Nippon Sheet Glass), followed by drying at room temperature and humidity. A band-pass filter (Toshiba Glass, G-55S) was used to extract green light from an Ushio Electric xenon arc lamp Model UXL-500D-O. The electrode potential was controlled with a TOHO Technical Research potentiostat, Model 2000, and an Ag/AgCl and a platinum wire served as the reference and the counter electrode, respectively.

Figure 1 depicts typical photocurrent response patterns from a bR-immobilized SnO_2 electrode at a neutral, low and high pH.[8] In what follows, the photocurrent by turning on and off of incident light is denoted, respectively, as the light-on and light-off photocurrent. As compared with the pattern at neutral pH (A), the photoresponse is reversed at low pH (B), and the light-off photocurrent significantly loses its intensity at high pH (C). The decay time of the light-on photocurrent is 4-6 ms at neutral and high pHs, and 7-10 ms at low pH. These are well in line with the kinetics of the bR photocycle. For any of these photocurrents, the action spectrum nearly coincided with the absorption spectrum of bR in suspension (data not shown).

The light-on photocurrent peak intensities are plotted in Figure 2 as a function of electrolyte pH. As seen the polarity is reversed at about pH 5.2. Since this is exactly the pH value at which the proton release/uptake is reversed,[9] the result in Figure 2 can be viewed as neatly reflecting the physiology of bR.

The ratio of the light-off to light-on photocurrent peak intensities, I_{off}/I_{on}, was systematically examined as a function of pH, and the results are depicted in Figure 3. The good coincidence between the plots of open circles (pH raised) and solid circles (pH lowered) demonstrates that the result in Figure 3
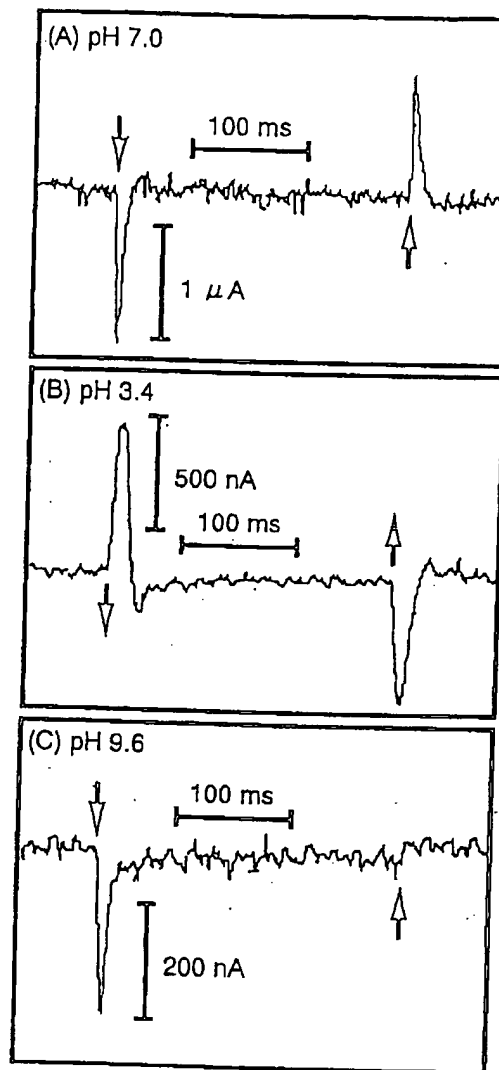

Figure 1. Photocurrent response from bR-SnO₂/electrolyte interface at various pH. (A) pH 7.0; (B) pH 3.4; (C) pH 9.6. The down and up arrows denote light on and off, respectively. Electrode potential, 0.0 V vs. Ag/AgCl. Light intensity, 1.3 × 10² mW cm⁻². Electrolyte, 0.1 M Na₂SO₄ with a mixture of buffers consisting of 10 mM sodium citrate, 10 mM sodium phosphate and 10 mM sodium borate. The pH value was controlled with HCl or NaOH.
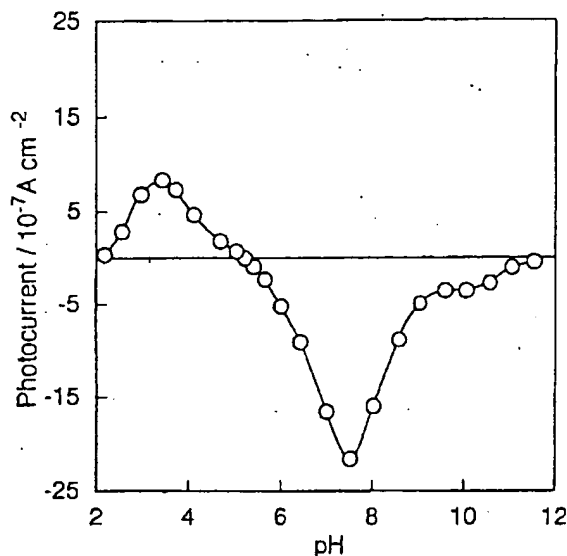
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Figure 2. pH-Dependence of peak photocurrent by turning the light on. Experimental conditions were the same as in Figure 1.
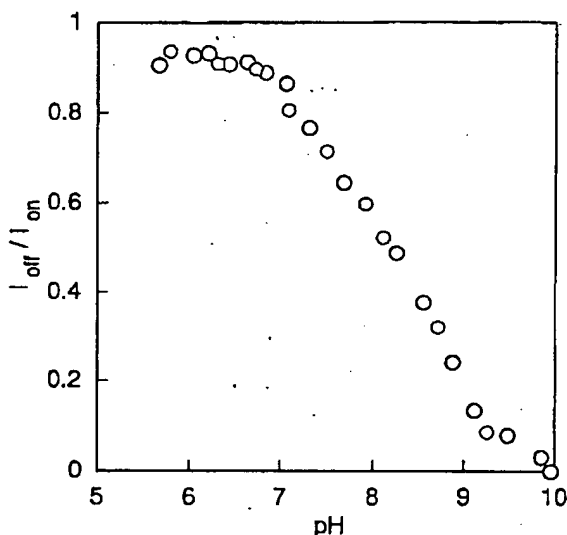

Figure 3. pH-Dependence of amplitude ratio of the photocurrent at light-off (I_{off}) to that at light-on (I_{on}). Electrolyte pH was raised with NaOH (O), or lowered with HCl (O).

reflects an inherent photochemistry in the bR molecules, and not their denaturation.

These findings could be rationalized by invoking the photocurrent generation mechanism, namely the shifting of proton dissociation equilibrium at an oxide electrode arising from proton ·release/uptake by bR molecules.[7] The latter, demonstrated by in · vivo studies,[2] is schematically illustrated in Figure 4. At neutral pH, the interfacial pH is lowered at the onset of illumination
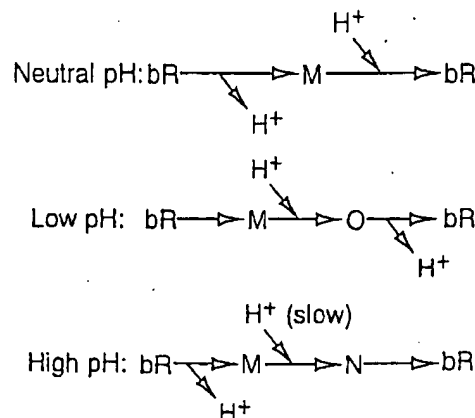

Figure 4. Simplified hR photocycles at neutral, low and high pH. M, N and O denote the intermediates in the bR photocycle. The Schiff base is deprotonated in the M intermediate and protonated in the other states. The conversion from N to O is associated with 13-cis to all-trans isomerization of the retinal chromophore.

because bR first releases a proton into the electrolyte solution, and this generates a cathodic capacitive current. This is followed by a photostationary state of the bR photocycle, where no charging current is to be expected. At the termination of illumination, the slower process (proton uptake) entails transiently, giving rise to an anodic capacitive current. At pHs below 5 the proton release/uptake sequence is reversed, and this is reflected in the photocurrent pattern reversal (Figures 1 and 2).

Although the proton release/uptake sequence is common in neutral and alkaline media, the later process (proton uptake) is naturally slowed at higher pHs,[10,11] and therefore the light-off peak photocurrent loses its intensity (Figures 1 and 3).

To summarize, the present results clearly indicate that the photoelectrochemical system could be a good probe to unravel the photochemistry and physiology of bR molecules.

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Ref. 2/   ○   ○

Mechanism of Photocurrent Generation from Bacteriorhodopsin on Gold Electrodes

Yoshitaka Saga and Tadashi Watanabe*

Institute of Industrial Science, University of Tokyo, Roppongi, Minato-ku, Tokyo 106-8558, Japan

Koichi Koyama and Tsutomu Miyasaka

Ashigara Research Laboratories, Fuji Photo Film Company, Ltd., Minami-ashigara, 250-0193, Japan

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Excitation of bacteriorhodopsin (bR) at an electrode–electrolyte interface generates transient photocurrents as evidenced by the turning of an incident light on and off. By use of a gold electrode as substrate, on which an oxide layer can be formed in a controlled manner, we have found two types of photocurrents, both originating in the excitation of bR. For the first type, arising most probably from the pH response of the surface oxide layer due to proton release/uptake by bR, the magnitude of photocurrent well paralleled the amount of surface oxide up to about one monolayer of Au_2O_3 and reached a maximum roughly equal to those observed on SnO_2 electrodes. The second type of photocurrent, being 3- to 4-fold smaller than the first one and essentially potential independent, arises presumably from simple charging, again through proton release/uptake by bR, of the electric double layer at the interface.

Introduction

Bacteriorhodopsin (bR), the sole protein in the purple membrane of *Halobacterium salinarium*, functions as a light-driven proton pump. The protein contains all-trans retinal as a chromophore bound to Lys-216 via a protonated Schiff base. Photoisomerization of the retinal from all-trans to 13-cis form triggers the transport of a proton from the cytoplasmic to the extracellular side, and the electrochemical potential thus built up across the membrane is then used for ATP synthesis.[1,2]

The proton transport in bR is a cyclic process. The reactions of retinal and protein during the photocycle, as confirmed by various spectroscopies, consists of interconversions of intermediate states denoted by J, K, L, M, N, and O.[3-6] In the transition from L to M, the proton on the Schiff base is transferred to Asp-85. A proton is subsequently released to the extracellular side, since the pK_a of the proton-releasing group in the extracellular domain is lowered by the protonation of Asp-85.[7-9] The decay of the M intermediate is accompanied by reprotonation of the Schiff base via Asp-96, and proton uptake by Asp-96 from the cytoplasmic side takes place in the transition from N to O.

Recently, photoelectric responses of bR at an electrode–electrolyte interface have been investigated,[10-16] where irradiation to bR-coated tin oxide (SnO_2) electrodes generated transient photocurrents by the turning on and off of the incident light. The origin of these photocurrents, however, has not been thoroughly understood yet.

Two groups proposed that the transient photocurrent is due to a pH change near the electrode.[14-16] Robertson and Lukashev examined the photocurrent responses of bR from the wild-type and a D96N mutant[14] where a nonprotonable asparagine replaced the protonable aspartate at position 96. The substitution of D96N

made the rate of proton uptake much slower. The photocurrent from the D96N mutant by turning the light off was very weak in the amplitude and the decay was very slow, probably because of the low proton concentration change near the electrode resulting in the slowing of proton uptake rate.

Wang et al. observed photoelectric responses from bR on indium tin oxide (ITO) electrodes under both pulsed and continuous light excitations.[15,16] The polarity of the differential photocurrents by continuous illumination (denoted components D1 and D2) was reversed at low pH, and this phenomenon was rationalized by invoking the proton release/uptake sequence in the bR photocycle, based on an assumption that the photocurrent originates in the pH response of the oxide electrode[17,18] due to proton release and uptake by bR. It is of much interest to substantiate this interpretation.

On a gold electrode, exhibiting a relatively wide oxide-free potential range, oxides can be formed in a controlled fashion.[19] This has incited us to study, in the present work, the photoelectrochemical responses of bR on gold electrodes, to find the existence of two types of photocurrents, one of which being indeed the pH response of the surface oxide, Au_2O_3.

Materials and Methods

Purple membranes were prepared from cultured *Halobacterium salinarium* S9 according to the methods of Oesterhelt and Stoecknius[20] and were suspended in pure water until use.

A gold plate of 99.95% purity (Nilaco) was washed first with hot methanol then treated with hot concentrated nitric acid and rinsed thoroughly with distilled water. A 50-μL aliquot of the bR suspension, with an optical density of 10 at 1-cm path length at 570 nm, was deposited on a 1-cm^2 area of the electrode and then dried at room temperature and humidity to obtain a bR-immobilized electrode. Tin oxide (SnO_2) electrodes were also used for control experiments, according to the procedures described elsewhere.[10]

The photocurrent measurement setup was essentially the same as the one reported previously.[21] An Ushio Electric 500 W xenon

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* To whom correspondence should be addressed: Institute of Industrial Science, University of Tokyo, Roppongi 7-22-1, Minato-ku, Tokyo 106-8558, Japan. Fax: +81-3-3401-5975. E-mail: watanabe@cc.iis.u-tokyo.ac.jp.

arc lamp Model UXL-500D-O served as the light source. Infrared radiation was removed with a 18-cm path length water cell, and a combination of Toshiba Glass cut filters O-56 and L-39 suppressed the short-wavelength light that tended to cause photocurrents due to excitation of the gold surface oxide itself.[19] For measurements of the photocurrent action spectra, the photon flux was determined by use of Koshin Kogaku interference filters (10-nm steps) and an Anritsu power meter Model MA9411.

A bR-immobilized electrode was mounted as a window (1 cm in diameter) of a photoelectrochemical cell. The supporting electrolyte was 0.1 M sodium sulfate in 10 mM phosphate buffer, pH = 7.2 (Jatron). The potential of the bR-immobilized electrode was controlled with a Toho Technical Research potentiostat Model 2000, with a Ag/AgCl and platinum wire as reference and counter electrodes, respectively. Photocurrents were measured with a Sony Tektronix oscilloscope Model TDS-340 after keeping the working electrode for 15 min at each potential. An NF Electric Instruments low-pass filter Model E-3201B was used in the measurements of photocurrent action spectra. Normally the measurements were repeated five times, and their averages are to be given below.

Each photocurrent measurement was followed by quantitation of surface oxide on the gold electrode from the area of an oxide reduction curve. A Hokuto Denko function generator Model HB-III was used to scan the potential at a rate of 10 mV s^{-1}, and current−potential curves were recorded on a Yokogawa X−Y recorder Model 3025. The electrode was held at an oxide-free potential (+0.1 V vs Ag/AgCl) before the photocurrent measurement at another potential.

Results

Photocurrent Patterns. Figure 1 depicts typical photocurrent response patterns from a bR-immobilized gold electrode held at +0.10, +0.75, and +0.90 V vs Ag/AgCl. At each potential, a cathodic transient current is observed by turning the light on, and an anodic transient current by turning the light off.[22] These patterns were essentially the same as those from bR-immobilized SnO$_2$ electrodes examined separately (Figure 6). However, the amplitude of the photocurrent from the bR-immobilized gold electrode at an oxide-free potential of +0.10 V is 3−4 times smaller than those from bR-immobilized SnO$_2$ electrodes.

A gold electrode undergoes surface oxidation substantially in a potential range positive of about +0.7 V vs Ag/AgCl. The photocurrents of Figure 1(b) and 1(c) are hence in such a potential range. The amplitude of the photocurrent at +0.90 V, Figure 1c, was a maximal one attained on gold electrodes, and was close to that generated from bR-immobilized SnO$_2$ electrodes (cf. Figure 6). For each trace in Figure 1, the action spectrum of the peak photoresponse was practically identical with the absorption spectrum of bR in suspension, as is seen in Figure 2. This indicates that the photocurrent results from excitation of bR on the electrode.

Photocurrent vs Surface Oxide. Under anodic polarization, a small stationary anodic photocurrent was observed even in the absence of bR. This is a photoelectrochemical response of gold oxide working as an n-type semiconductor electrode, as was reported in detail previously.[19]

On an anodic scan, surface oxidation of gold is seen to start on a small scale from around +0.5 V vs Ag/AgCl and substantially from +0.70 V, and pass through a maximum at +1.05 V. On the reverse scan, the oxide reduction curve peaks at around +0.50 V. In Figure 3 the oxide formation charge, obtained from integration of the reduction current−potential
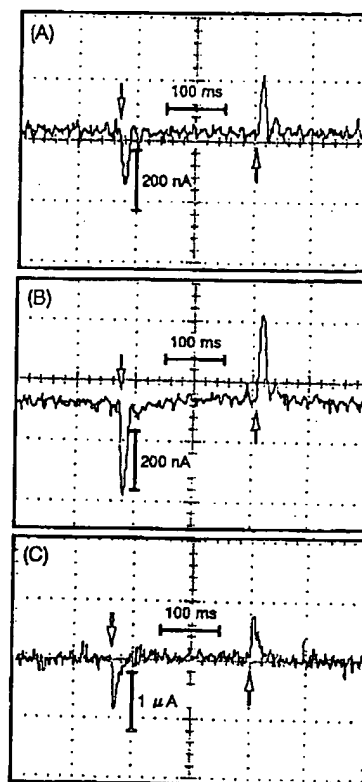

Figure 1. Photocurrent response patterns for bR on a gold electrode at three potentials. (A) +0.10, (B) +0.75, (C) +0.90 V vs Ag/AgCl. The arrows denote turning on and off of light. Light intensity, 40 mW cm^{-2}. Electrolyte, 0.1 M Na$_2$SO$_4$ in 10 mM phosphate buffer of pH 7.2.

curve, is compared with the amplitude of the peak photocurrent as a function of electrode potential. Evidently there are two distinct branches in the photocurrent: the one growing in parallel with the progress of surface oxidation, but tending to level off at potentials beyond about +0.85 V, and the other (below +0.7 V) being independent of surface oxidation.

For the potential range where surface oxidation was clearly noted on the reduction curve, the peak photocurrent is compared in Figure 4 with the oxide formation charge. The amplitude of the photocurrent well parallels the oxide formation charge up to around 300 μC cm^{-2} and levels off beyond this. The thickness of gold surface oxide can be estimated from the oxidation charge as follows. As a first approximation, the surface layer was assumed to be the main oxide, Au$_2$O$_3$,[19] for which the density has been evaluated to be 11 g cm^{-3}.[23] The roughness factor of the electrode surface is tentatively assumed to be 2 in view of literature values,[24−26] though its reliable determination is generally difficult.[26] By use of these values and the molar charge of 6F, the monolayer of Au$_2$O$_3$ corresponds to a charge density of ca. 300 μC cm^{-2}. Therefore, Figure 4 demonstrates that the bR-derived photocurrent is roughly proportional to the amount of surface gold oxide up to a monolayer of the latter.

Higher Potential Range. Two factors could be envisaged as the cause for the photocurrent leveling off on gold electrodes at higher potentials. One is when a monolayer of Au$_2$O$_3$ is enough to exhibit the pH response of the oxide,[17,18] and the other factor is a potential-induced denaturation of bR. The latter possibility was examined by comparing the photocurrent at an
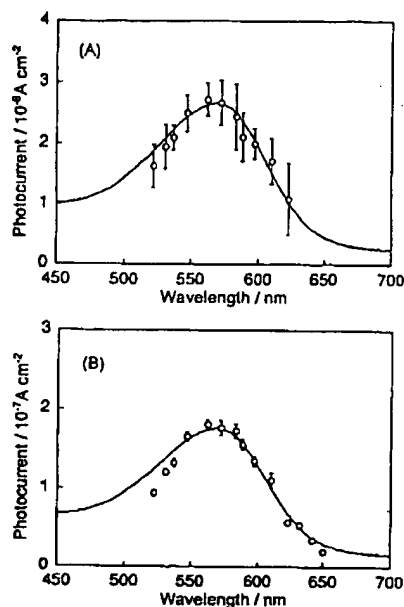
Saga et al.

Figure 2. Action spectra of the peak photocurrent from bR on a gold electrode. (A) +0.10, (B) +0.80 V vs Ag/AgCl. Incident monochromatic photon flux, 1.19×10^{16} cm^{-2} s^{-1}. The solid curve represents the absorption spectrum of bR in suspension.
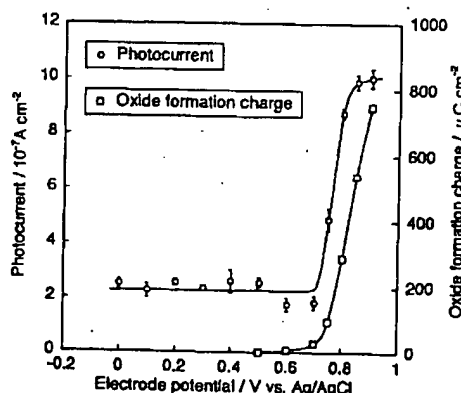

Figure 3. Dependencies of the photocurrent density from bR on a gold electrode and the oxide formation charge on the electrode potential.

oxide-free potential (+0.1 V) before and after a journey to higher potentials. The result is displayed in Figure 5 in the form of I/I_0 against the potential, where I_0 and I denote the photocurrent before and after the potential journey, respectively. As is seen, the photoactivity remains nearly constant at 1.0 ± 0.1, and hence the denaturation of bR is negligible up to a potential of +0.8 V, beyond which a denaturation appears to take place probably due to irreversible oxidation of bR.

Photocurrent from bR on SnO$_2$ Electrodes. Figure 6 depicts a typical photocurrent response from a bR-immobilized SnO$_2$ electrode at 0.0 V vs Ag/AgCl. The amplitude and kinetics are similar to those for a maximal photoresponse attained on gold electrodes (Figure 1 c). The peak photocurrents from a bR-immobilized SnO$_2$ electrode are plotted in Figure 7 against the electrode potential. The amplitude is almost independent of electrode potential from 0.0 V to +0.8 V, and the decrease in the photocurrent intensity at above +0.8 V is similar to that in Figure 5, probably due to the denaturation of bR.
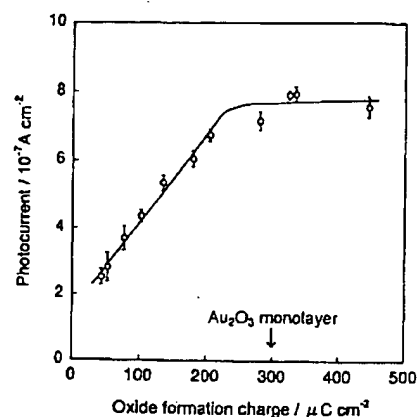

Figure 4. Photocurrent density as a function of the oxide formation charge on gold electrodes.
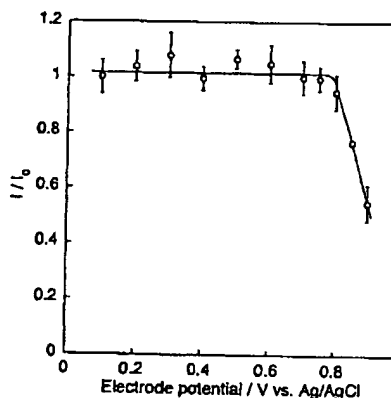

Figure 5. Ratio of the photocurrent density at +0.1 V after a potential journey to a positive potential on the abscissa (I) to that before the potential journey (I_0).
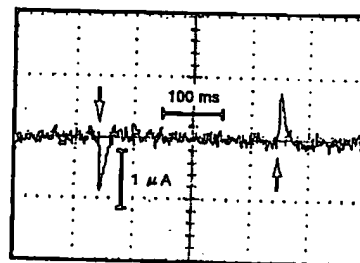

Figure 6. Photocurrent response patterns for bR on an SnO$_2$ electrode at 0.0 V vs Ag/AgCl. The arrows denote turning on and off of light.

Discussion

Excitation of bR on gold electrodes generates transient photocurrents by turning on and off of the incident light, in the same manner as on SnO$_2$ and ITO electrodes.[14-16] We were able to distinguish between two types of photocurrents on gold. One type commences to increase at around +0.7 V vs. Ag/AgCl, well in parallel with the start of surface oxidation, and is saturated beyond about +0.85 V. The saturation comes either from the sufficient thickness (more than a monolayer) or from some oxidative denaturation of bR at high potentials.

The mechanism of transient photocurrent generation from bR on a metal oxide electrode could be envisaged as shown in
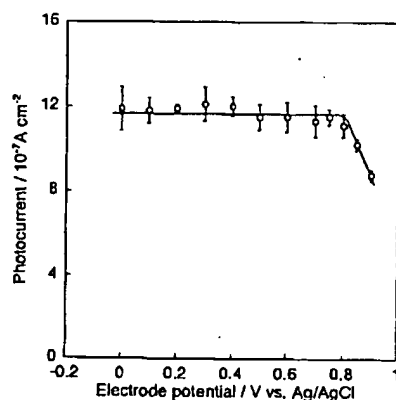
Figure 7. Potential dependence of the photocurrent density from bR on an SnO₂ electrode.
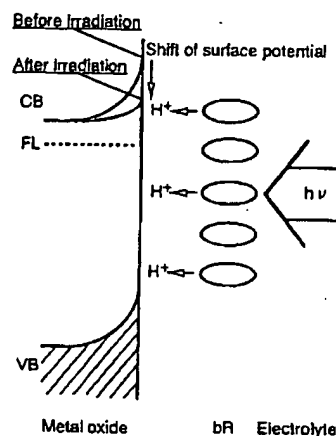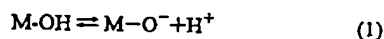

Figure 8. Mechanism of the transient photocurrent generation from bR on an oxide-covered metal electrode. CB, VB, and FL denote the conduction band, valence band, and Fermi level, respectively.

Figure 8, where the oxide is viewed as an n-type semiconductor electrode, as is the case for gold oxides.[19] On the surface of a semiconductor electrode in contact with an aqueous electrolyte, a proton dissociation equilibrium

$$M\text{-}OH \rightleftharpoons M\text{-}O^- + H^+ \tag{1}$$

is established for the surface hydroxide (M—OH). Protons released from excited bR in the interfacial region should shift the equilibrium to the left-hand side, and this shift is reflected in the anodic shift of the so-called flatband potential. In an ordinary situation where the semiconductor electrode is connected only to a reference electrode, the energy bands of the semiconductor are shifted anodically (downward in Figure 8), with no change in the band bending profile just beneath the surface. However, under potentiostatic conditions as in the present measurements, the potential shift can take place only on the surface, diminishing the band bending in the space charge layer. Such a change is equivalent, in terms of the potential profile in the space charge layer, to a cathodic polarization of the semiconductor electrode, giving rise to a transient capacitive (charging) current. The transient photocurrent at the onset of irradiation can be interpreted in this manner. Indeed, when a millivolt-level negative potential step was given to a bR-free gold electrode at a potential of +0.9 V vs Ag/AgCl in the dark,
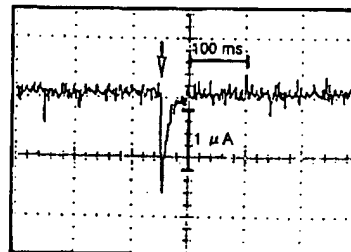

Figure 9. Transient current resulting from a −1 mV potential step applied to a gold electrode held at +0.9 V vs Ag/AgCl with no bR deposited. Electrolyte: 0.1 M Na₂SO₄ in 10 mM phosphate buffer of pH 7.2.

a capacitive current was found to flow in the cathodic direction, as illustrated in Figure 9. The kinetics here (decay rate in particular) are almost the same as those of the transient photocurrents (Figure 1).

At the onset of irradiation, the interfacial pH is lowered because bR first releases a proton into the electrolyte solution, and this gives rise to a capacitive cathodic current as discussed above. This is followed by a slower process of proton uptake by bR, which recovers the interfacial pH to the initial value. Hence no current flows during continuous irradiation (for ca. 250 ms in the present experiments) where the same amount of proton is released and taken up in the bR photocycle. At the termination of irradiation, the slower process (proton uptake) entails transiently, giving rise to an anodic capacitive current. This view is corroborated by the reported photocurrent polarity reversal at lower pHs, i.e., generation of an anodic photocurrent at the onset of irradiation and a cathodic photocurrent by turning off the light,[15,16] and by invoking the reversal of the proton release/uptake sequence in the bR photocycle.

Another type of photocurrent was observed in this work, at potentials where practically no oxide is present on the gold electrode and hence we could not expect the proton dissociation equilibrium shift on the surface oxide. Though the magnitude of this photocurrent is essentially potential independent, in contrast to that at > +0.7 V, its kinetics are very similar to those found on oxide-covered electrodes. We tentatively interpret this as arising from simple charging of the electric double layer near the electrode surface due to increase/decrease in the positive charge (proton) due to photoexcitation of bR. Further work is needed to identify the exact origin of this type of photoresponse.

With experimental setups essentially different from the one used here, the flash-induced photoelectric signals from oriented purple membranes, probably due to internal charge movement associated with conformational change of bR and/or external charge movements such as proton pumping, have been detected.[27-42] The signal has at least three components with duration times lasting from picoseconds to milliseconds. The first component (B1), with a rise time shorter than 100 ps,[30,31] is thought to originate in the charge movement induced by all-trans to 13-cis isomerization of retinal.[32-34] The second component (B2), with a lifetime of 40−100 μs, is correlated with the L−M transition,[32,35-39] or partially to a proton movement from the Schiff base to Asp-85. Misra envisaged a contribution of proton release to the B2 component[40] in addition to the proton movement inside bR, as had been suggested before.[41,42] A much slower component (B3) has been observed in the millisecond regime.[15,16,29,36,38] Though the origin of this component has not been fully understood, Wang et al.[15,16] proposed that this component might correspond to the transient photocurrent under continuous excitation, namely, to the proton

release/uptake by bR. This interpretation may hold also for the photocurrents observed in the present work, since the circuit time constant (ca. 3 ms) of the potentiostatic system does not permit detection of sub-millisecond events at all.

In two-electrode systems capable of detecting rapid signals, a high degree of orientation of bR molecules is required for the observation of photoelectric responses arising from the charge polarization and protein relaxation of bR. In the present experiments, however, the orientation of bR molecules was not controlled on the surface, but the photocurrent was highly rectified. The polarity of the photocurrent would not depend on the membrane orientation, as long as the photoresponse arises, as discussed above, from the interfacial pH change due to proton release/uptake by the bR molecules. Indeed, in experiments where the photoresponse behaviors were compared between a membrane with the cytoplasmic side facing the electrode and another membrane with the extracellular side facing the electrode, the photocurrent polarity was common, and its magnitude alone depended on the membrane orientation.[13]

The transient photocurrent from bR at an electrode–electrolyte interface as employed here, revealing the activities of the proton release and uptake sites,[43] is different from the rapid photoelectric signal from oriented purple membranes by flash excitation, originating in the charge displacement within the bR molecules. These two photoelectric responses hence deliver different pieces of information about the proton-transfer processes in bR.

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Ref.30

Lifetime of M Intermediate in the D96N Mutant of Bacteriorhodopsin Determined by a Photoelectrochemical Method

Koichi Koyama,* Tsutomu Miyasaka, Richard Needleman,[†] and Janos K. Lanyi[††]
Ashigara Research Laboratories, Fuji Photo Film Co., Ltd., Minamiashigara, Kanagawa 250-0193
[†]*Department of Chemistry and Molecular Biology, Wayne State University, Detroit, MI, U.S.A.*
[††]*Department of Physiology and Biophysics, University of California, Irvine, CA, U.S.A.*

The recombinant D96N mutant of bacteriorhodopsin (bR) was immobilized on SnO_2 electrode that contacts an aqueous electrolyte. By laser pulse excitation, photocurrent behavior of D96N was analyzed to evaluate the lifetime of the M intermediate involved in proton transfer reactions. The half-life of the M intermediate in the photocycle was about 5 s, which is 10^3-fold longer than that of wild-type and shows the crucial role of D96 in the uptake of protons.

The photocycle of bacteriorhodopsin (bR), the light-driven proton pump in *Halobacterium salinarum*, comprises a series of intermediates that is initiated by all-trans to 13-cis isomerization of retinal. The latter causes deprotonation of the retinal Schiff base, which stimulates proton release to the extracellular side. The Schiff base is subsequently reprotonated by D96 that is located at the cytoplasmic side.[1]

The importance of D96 in the proton uptake process was first pointed out by Mogi et al.[2] They prepared a site-specific mutant by replacing D96 with Asn (D96N) and showed that D96N has no proton pumping activity due to accumulation of the M intermediate[3] in lack of proton donor. The decay of the M intermediate therefore correlates to the rate of proton uptake and reprotonation of the Schiff base.[4,5]

We have previously demonstrated[6,7] that the photocurrent behavior of bR at the electrode/electrolyte interface is a useful probe for the molecular mechanisms in the proton release and uptake processes that link to the M formation. In this paper, we report a photoelectrochemical approach to determine the lifetime of the M intermediate and the role of D96 in proton uptake reaction by using the mutant D96N.

D96N was expressed in *Halobacterium salinarum* by the procedure as described before.[9] The D96N mutant was suspended in pure water for film preparation. Construction of the electrochemical cell that immobilizes D96N basically follows our previously reported method.[8]

Light source was a 150 W xenon arc lamp and used with an infrared cut-off filter (Toshiba IRA-05) and a band-pass filter (HOYA G550). Photocurrent was measured with a circuit comprising an operational amplifier that converts a small transient current into a dc voltage; the signal was recorded on a Gould Model 420 and/or Hewlett Packard Model 54520C digital storage oscilloscope.

Figure 1 shows the profile of photoelectric response obtained for D96N. The transient positive (cathodic) and negative (anodic) signals by turning-on and off of light, respectively, represent capacitive currents induced by a surface potential shift at SnO_2 electrode, caused by the proton release and uptake reactions of the protein. The suppression of the negative response results from the lack of proton uptake ac-

tivity in D96N. This, as well as, observed slow decay of the positive response compared to the wild-type[8] are assumed to give a long lifetime for the M intermediate (13-cis state) that lasts until the proton uptake recovers the initial state (all-trans state) of the D96N photocycle. However, the lifetime of M in D96N is not clear because lack of kinetic information on the recovery of the initial state.
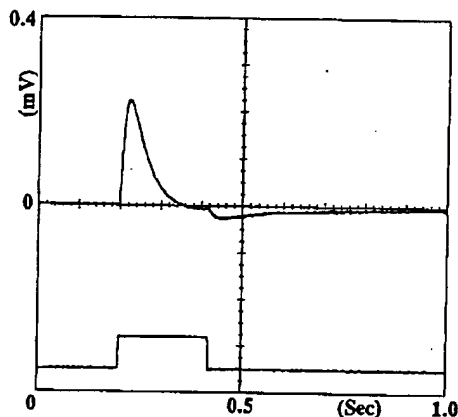

Figure 1. Response profile of D96N immobilized at the SnO_2/electrolyte (0.1 M KCl, pH 8.0) interface in an electrochemical cell. The cell was irradiated 0.25 s with continuous green light supplied by a 150 W xenon arc lamp. Light intensity pattern is given below the response. Photocurrent was converted into dc voltage (ordinate).

To estimate the lifetime of the M intermediate in D96N, we carried out kinetic investigations by laser pulse excitation. The D96N-immobilized electrochemical cell was excited with a 7-ns pulse (<10 mJ/cm²) of the 532 nm second harmonics supplied by a Nd-YAG laser (Continuum Surelite I). Following the initial pulse excitation that causes substantially total conversion to the M intermediate, the cell was pulse-excited again with different time lags which allows for diminution of the intermediate. Because the M intermediate only absorbs in blue region (<500 nm, maximum at 412 nm), the response amplitude exhibits the population of the initial all-trans state D96N. We have previously confirmed that only all-trans state generates photocurrent.[10]

Figure 2 shows the response data collected with different time intervals (0.1 to 30 s) between two excitations. At a time interval of 0.1 s, a majority of D96N stays at M, which is detected with the 412 nm absorption of M. The response amplitude recovers with an increase in the time interval, i.e.,

with the decay of M and eventually the half-lifetime of M decay is around 5 s and completion of decay to recover the initial D96N takes more than 30 s. In contrast, the wild-type bR takes less than 10 ms to complete the photocycle. This shows that the D96 displacement with N retards the decay of the M intermediate by more than 10^3 times that of wild-type. We confirmed that the addition of azide (up to 100 mM) to electrolyte no longer retards the M decay of D96N and the recovery occurs even at interval of 0.1 s, which coincides with the results reported by Tittor et al.[11] and supports the fact that azide can participate in proton shuttling between the Schiff base and the bulk aqueous phase.[12]
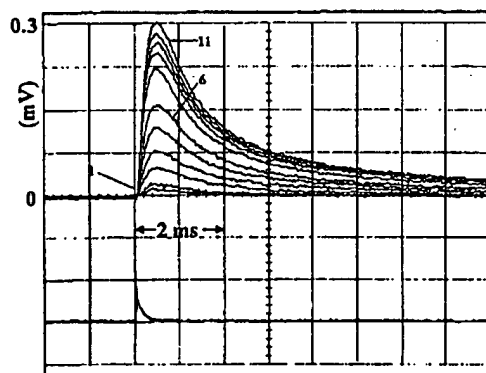

Figure 2. Dependence of the D96N response (second excitation) on the time interval after the first excitation, measured at pH 8.0. The profiles 1 through 10 were obtained at intervals of 0.1, 0.2, 0.5, 1, 3, 5, 10, 15, 20, 30 s after first excitation and 11 is the response of the first excitation. Laser pulse pattern is given below the response.

In Figure 3, it is shown that the M decay in the wild-type undergoes retardation when measured at pH 10. Similar to the case in D96N, wild-type bR was found to undergo retardation of the M decay by raising pH of the electrolyte. Knowing that the pKa of D96[13] is >11 and, at pH 10, D96 stays protonated and functions as proton donor, this phenomenon may not be due to deprotonation of D96. We consider that the low proton concentration in the bulk electrolyte is responsible for the observed retardation, that is, inefficiency in proton pumping.
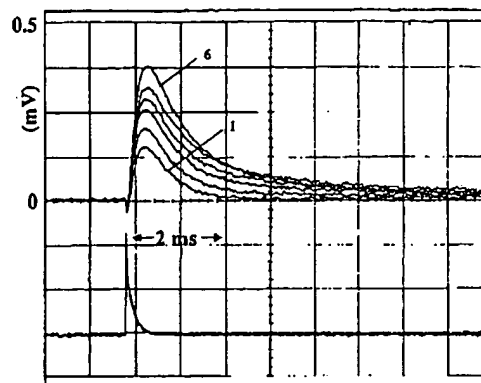
We determined the lifetime of the M intermediate in D96-

Figure 3. Dependence of wild-type response (second excitation) on the time interval, measured at pH 10. The profiles 1-5 and 6 correspond to 0.1, 0.2, 0.5, 1, 2 s and the first excitation, respectively. Laser pulse pattern is given below the response.

lacking mutant of bR and showed that D96N normally releases protons by illumination but takes much time until the next proton release occurs. Our photoelectrochemical evaluation demonstrates that D96 in the cytoplasmic half of bR is crucial to effect the reprotonation of the retinal Schiff base and play the key role in controlling the lifetime of the M intermediate.

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